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Assessing phage therapy against *Pseudomonas aeruginosa* using a *Galleria mellonella* infection model

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ABSTRACT

The *Galleria mellonella* infection model was used to assess the *in vivo* efficacy of phage therapy against laboratory and clinical strains of *Pseudomonas aeruginosa*. In a first series of experiments, *Galleria* were infected with the laboratory strain *P. aeruginosa* PAO1 and were treated with varying multiplicity of infection (MOI) of phages either 2 h post-infection (treatment) or 2 h pre-infection (prevention) via injection into the haemolymph. To address the kinetics of infection, larvae were bled over a period of 24 h for quantification of bacteria and phages. Survival rates at 24 h when infected with 10 cells/larvae were greater in the prevention versus treatment model (47% vs. 40%, MOI = 10; 47% vs. 20%, MOI = 1; and 33% vs. 7%, MOI = 0.1). This pattern held true when 100 cells/larvae were used (87% vs. 20%, MOI = 10; 53% vs. 13%, MOI = 1; 67% vs. 7%, MOI = 0.1). By 24 h post-infection, phages kept bacterial cell numbers in the haemolymph 1000-fold lower than in the non-treated group. In a second series of experiments using clinical strains to further validate the prevention model, phages protected *Galleria* when infected with both a bacteraemia (0% vs. 85%) and a cystic fibrosis (80% vs. 100%) isolate. Therefore, this study validates the use of *G. mellonella* as a simple, robust and cost-effective model for initial *in vivo* examination of *P. aeruginosa*-targeted phage therapy, which may be applied to other pathogens with similarly low infective doses.

1. Introduction

Multidrug-resistant bacterial pathogens pose an ever-increasing threat to human health. This problem is in part due to a lack of novel antibiotics approved for use over the last few decades, resulting in an urgent need to identify new avenues for treating bacterial infections, especially those caused by Gram-negative pathogens [1]. *Pseudomonas aeruginosa* is an opportunistic pathogen that is a leading cause of infection among burn victims and patients with cystic fibrosis (CF). It is also responsible for a large number of healthcare-associated infections. To make matters worse, *P. aeruginosa* is associated with hypermutability and, due to high antibiotic selective pressure, has given rise to the emergence of multidrug-resistant strains in the population; thus, concerns about available effective treatments are growing [2,3]. In the UK, resistance to two or more antibiotics among *P. aeruginosa* isolated from the lungs of CF patients has risen to 40% [4]. This is a worrying statistic as colonisation of the CF lung with *P. aeruginosa* is a predictor of poor prognosis and is associated with a two- to three-fold increased risk of death over an 8-year period [5]. For this reason, novel anti-infectives are needed.

Facing such a scenario, interest in phage therapy in Western society has experienced a resurgence after research into this area fell out of favour following the discovery of antibiotics. Bacteriophages (or phages) are viral particles able to infect bacterial cells with high specificity, taking over cellular function to replicate their genomes. Upon maturation, the bacterial cell wall is lysed to release viral progeny.

Phage therapy can be broadly subdivided into four main categories [6]: (i) conventional phage therapy using mainly lytic phages to lyse target bacterial species; (ii) modified phage therapy using genetically altered phages with favourable properties, such as non-lytic replication to avoid the possibility of endotoxic shock when bacterial cells are lysed; (iii) treatment with enzymes derived from phages, such as administration of endolysins to selectively

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degrade the bacterial peptidoglycan cell wall; and (iv) the concept of combination therapy with phages and antibiotics, where phages exhibit properties to degrade polysaccharide components of biofilms therefore allowing antibiotics to penetrate and elicit an action [7].

Although *in vitro* systems allow for a reductionist approach to examining phage interactions with target bacteria, they do not take into account a more complex *in vivo* system. Mammalian models are an excellent means of testing phage therapy but require ethical approval, significant infrastructure and funds. The *Galleria mellonella* model fills the void between these two systems, providing a cheap, reliable and ethics-free system for testing novel antimicrobials [8]. Here we describe the first use of the *G. mellonella* model to evaluate the efficacy of phage therapy both for treatment and prophylaxis of *P. aeruginosa* infection.

2. Materials and methods

2.1. Bacterial strains and preparation of inoculum

Phage therapy was assessed using *P. aeruginosa* PAO1 and two low-passage clinical isolates (PA45291 and BC09007) isolated from bacteraemia and CF samples, respectively. Bacteria were grown to mid-log phase in Luria–Bertani (LB) broth (Sigma-Aldrich, Dorset, UK) and were washed once in phosphate-buffered saline (PBS). Cells were re-suspended in PBS to a final concentration of 1×10^8 CFU/mL and were diluted accordingly in PBS to the required inoculum size for each experiment.

2.2. Phage cocktail preparation and titration

All six distinct phages were propagated on *P. aeruginosa* PAO1 strain and were combined to establish a cocktail suspension. The genomic sequence of the six phages can be found in the National Center for Biotechnology Information (NCBI) GenBank database under accession nos. KR054028–KR054033, with a full description of the phages detailed elsewhere (submitted). Briefly, 100 μL of phage lysate and 100 μL of host growing culture were mixed and left for 5 min at room temperature. Following incubation, 3 mL of LB soft agar (Sigma-Aldrich) containing 0.65% bacteriological agar (Sigma-Aldrich)was added and poured onto agar plates. Following overnight incubation at 37 °C, plates displaying confluent lysis were selected and 3 mL of SM buffer [5 M NaCl, 1 M $MgSO_4$, 1 M Tris–HCl (pH 7.5), 0.01% w/v gelatine] and 2% (v/v) chloroform (Sigma-Aldrich)were added before incubation at 37 °C for 4 h. High-titre phage solution was removed from the plates, centrifuged ($8000 \times g$, 10 min) to remove cell debris and then filter-sterilised (pore size, 0.22 μm). A polyethylene glycol (PEG) (Sigma-Aldrich) purification step was further added to remove any possible bacterial debris from the suspensions. Briefly, 10% (w/v) PEG (*MW* 8000) was added to the lysate and left overnight at 4 °C. The next day, the solution was centrifuged (4000 rpm, 30 min) to obtain a PEG–phage pellet. The pellet was re-suspended gently in 1 mL of SM buffer and was vortexed thoroughly. The final solutions were stored at 4 °C. All of the necessary dilutions were performed in SM buffer. For titration of the bacteriophage content in the haemolymph, a similar methodology to the propagation was followed. Several dilutions were mixed with host bacterial cells and 3 mL of soft agar was added and poured onto agar plates. Following overnight incubation, plaques were counted to determine the phage titre.

2.3. Galleria mellonella phage therapy assay

Larvae of *G. mellonella* were obtained from Livefood UK Ltd. (Rooks Bridge, UK). Larvae were stored at 4 °C and were used within 1 week. A modified methodology developed by Peleg et al. was used to infect each *G. mellonella* [8]. Briefly, *G. mellonella* were surface-sterilised with a FASTAID pre-injection swab (Sigma-Aldrich) containing 70% ethanol (Sigma-Aldrich). Using a pair of tweezers, each *G. mellonella* was restrained and, using a 26 G Terumo syringe (VWR International, Lutterworth, UK), 10 μL of inoculum containing either 100 or 10 cells of *P. aeruginosa* was delivered into the larval haemolymph behind the last proleg. For the treatment model, phage suspension was delivered behind the last proleg on the opposite side to the bacterial injection site 2 h post-infection, and for the prevention experiment phage suspension was given 2 h pre-infection. All experiments used 15 larvae per treatment. A positive control group (larvae infected and treated with PBS solution) and two negative control groups (one group injected with PBS only, assessing the impact of any negative effect from the injection process; and one group injected with phage suspension only, assessing toxicity of the phage cocktail) were also included. Larvae were placed into Petri dishes and were incubated at 37 °C. *G. mellonella* were examined hourly after 15 h post-infection and were recorded as dead when they did not move in response to touch.

2.4. Bleeding larval haemolymph

The prevention model was used to follow the kinetics of bacteria and phage interactions within the larval haemolymph over time. The phage cocktail, or PBS, was administered 2 h prior to infection and the phage titre was initially quantified within the haemolymph at the time of infection (time zero). *G. mellonella* were infected with 100 cells of *P. aeruginosa* PAO1 and at 8 h and 24 h three *G. mellonella* were sacrificed and bled following incision made with forceps to quantify phage and *P. aeruginosa* both in phage- and PBS-treated *G. mellonella*. Titrations of haemolymph were made in SM buffer for phage counts. Quantification of PAO1 was done by preparing serial dilutions of haemolymph in 10 mM ferrous ammonium sulfate (FAS) (Sigma-Aldrich) for inactivation of extracellular phage. Bacteria were enumerated by total viable count of FAS dilutions onto LB agar. Inactivation of phages by FAS was confirmed prior to experimental procedure (data not shown). To rule out the possibility of PAO1 evolving phage resistance during *in vivo* infection, re-isolated PAO1 were subjected to plaque assay to confirm susceptibility.

2.5. Statistical analysis

Kaplan–Meier survival curves and log-rank (Mantel–Cox) statistical test were performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA).

3. Results

3.1. Treatment of infection

In this study, two models of phage and infection interactions were examined. The first was a treatment whereby *G. mellonella* was infected with either 10 or 100 cells of *P. aeruginosa* PAO1 and left to allow an infection to establish for 2 h. Varying multiplicity of infection (MOI) of phage were then administered and mortality was observed over 48 h. No mortality was recorded in the PBS controls. However, *G. mellonella* treated with PBS died quicker when infected with 100 cells compared with 10 cells. Administration of phage, displaying lytic activity against PAO1 *in vitro*, prolonged the survival of *G. mellonella* in a dose-dependent manner, but 0% survival was eventually seen in all groups by 30 h (Fig. 1A and B). At 24 h there was 100% mortality in the infected and untreated *G. mellonella*, but 40% survival for those infected with 10 cells and treated with a MOI of 10 compared with 20% survival in those infected with

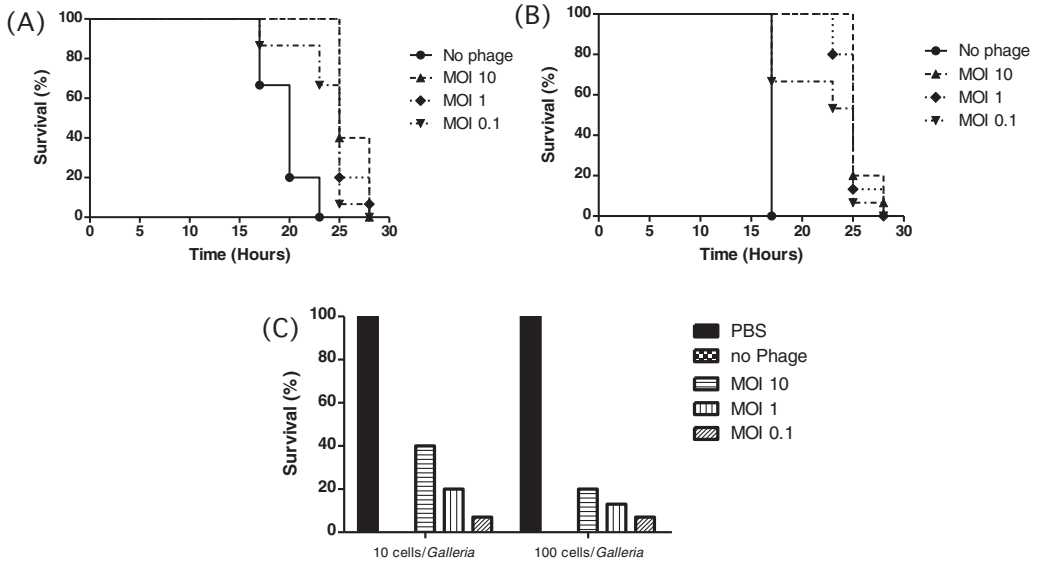

Fig. 1. Kaplan–Meier survival curves of *Galleria mellonella* infected with (A) 100 cells or (B) 10 cells of *Pseudomonas aeruginosa* PAO1 and treated with phage at varying multiplicity of infection (MOI) 2 h post-infection. (C) Percentage of *G. mellonella* survival at 24 h. PBS, phosphate-buffered saline.

100 cells at the same MOI (Fig. 1A and B). A statistically significant difference was seen between the survival curves as determined by log-rank (Mantel–Cox) test ($P < 0.0001$).

3.2. Prevention of infection

The second model examined the effect of prevention of infection whereby *G. mellonella* was given a prophylactic dose of phage 2 h prior to infection with *P. aeruginosa* PAO1. Similarly to the treatment experiment, *G. mellonella* infected with 100 cells died quicker than those infected with 10 cells when given PBS 2 h before infection (Fig. 2A and B). At 24 h, survival ranged from 80% in *G. mellonella* infected with 100 cells and given a MOI of 100 to 35% in those given a MOI of 0.1 (Fig. 2A). Survival ranged from 90% to 60% in *G. mellonella* infected with 10 cells and given MOIs of 100 and 1, respectively (Fig. 2B). A statistically significant difference was seen between the survival curves as determined by log-rank (Mantel–Cox) test ($P < 0.0001$).

3.3. Kinetics of Pseudomonas aeruginosa infection and effect of phage treatment

To understand the kinetics of a *P. aeruginosa* infection within *G. mellonella*, larvae were infected with 100 cells using the prevention model of infection. Bacteria and phage were quantified at set time points by bleeding the haemolymph. Recovered volumes of haemolymph ranged from 20 μL to 40 μL, but numbers were standardised upon analysis. No endogenous *P. aeruginosa* or phage with lytic activity against *P. aeruginosa* PAO1 were detected in the uninfected controls. For *G. mellonella* that were given *P. aeruginosa* PAO1 only, the number of cells isolated from the haemolymph increased over the duration of the experiment. By 24 h, all *G. mellonella* were

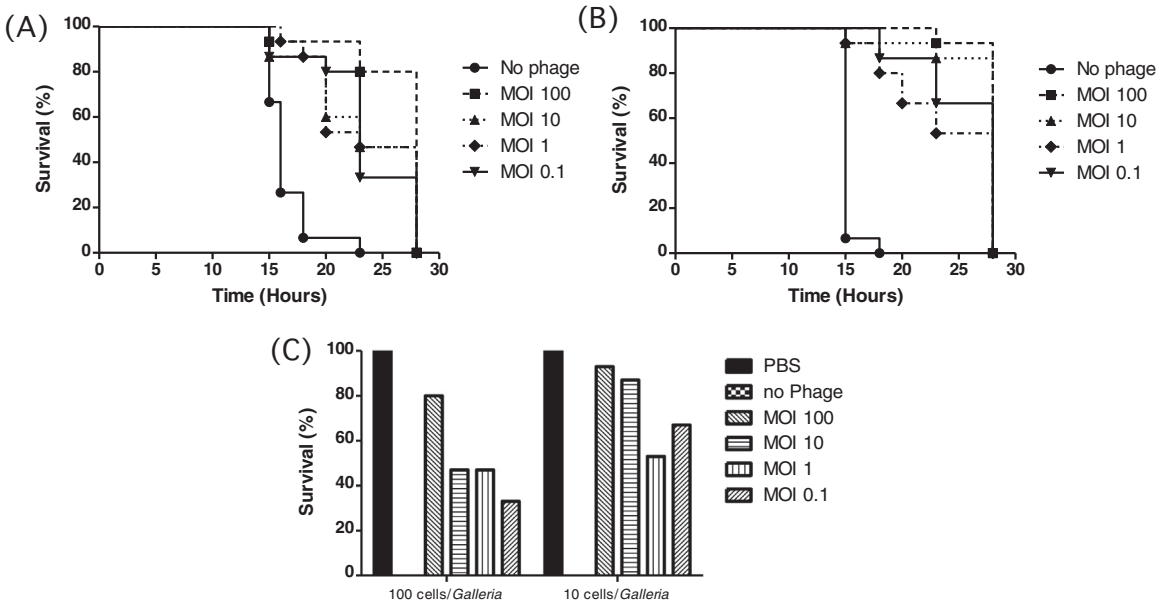

Fig. 2. Kaplan–Meier survival curves of *Galleria mellonella* infected with (A) 100 cells or (B) 10 cells of *Pseudomonas aeruginosa* PAO1 and pre-treated with phage at varying multiplicity of infection (MOI) 2 h pre-infection. (C) Percentage of *G. mellonella* survival at 24 h. PBS, phosphate-buffered saline.

dead and number of *P. aeruginosa* were in the order of 10^8 CFU/mL. The second group of *G. mellonella* was given a prophylactic dose of phage 2 h prior to infection and then phage and bacteria were quantified over the course of the infection. The number of *P. aeruginosa* PAO1 was comparable with that of the non-treated *G. mellonella* after 8 h of infection, but were three orders of magnitude less at 24 h compared with the non-treated *G. mellonella*. These *G. mellonella* were alive at 24 h. The number of phages increased over the duration of the infection, reaching a peak titre at 24 h of 10^8 PFU/mL.

3.4. *Low-passage clinical isolates of* Pseudomonas aeruginosa

To validate the model of phage therapy with *P. aeruginosa*, we sought to test the model with low-passage clinical strains isolated from patients with bacteraemia or CF (Fig. 4A and B). With the PA45291 bacteraemia strain, all infected *G. mellonella* were dead by 24 h, whereas there was 60% survival at 28 h in the group treated with phage at a MOI of 10. When *G. mellonella* were infected with the BC09007 CF strain there was little mortality at 24 h (90%) when given PBS as treatment, but there was 100% survival in the phage-treated group. By 40 h, all *G. mellonella* were then dead.

4. Discussion

To avoid a scenario whereby society is plunged back into a pre-antibiotic era, there is an urgent need to identify novel antibacterial agents. Phage therapy offers a novel non-antibiotic approach to help in this battle. Phage therapy offers a different mode of action compared with antibiotics and therefore antibiotic-resistant organisms can still be susceptible to phages. In addition, phages are highly selective and will therefore not wipe out the host microbiota, unlike antibiotics, as well as being deemed safe in trials [9–11].

The *G. mellonella* infection model provides a system that can bridge the gap between *in vitro* studies and more advanced mammalian studies, giving initial proof-of-principle data. Mammalian models are crucial for testing the efficacy of phages prior to human trials, but drawbacks include the need for sufficient infrastructure, substantial costs as well as the need for ethical approval. *G. mellonella* larvae have been used to examine numerous host–pathogen interactions ranging from studies of pathogenicity to antimicrobial activity, with a small number of these examining the potential for phage therapy [12–14].

The *P. aeruginosa* PAO1 strain proved to be highly virulent, with only 10 cells per *G. mellonella* required to result in mortality at 24 h. This is a very low infective dose in this model, with organisms such as *Staphylococcus aureus* requiring 10^5–10^6 cells/*G. mellonella* for mortality, *Acinetobacter baumannii* requiring $>10^4$ and *Helicobacter pylori* requiring 10^6–10^7 cells for establishment of infection [15–17]. This low infectious dose is of particular interest as it reduces the chances of endotoxic shock due to rapid lysis of high numbers of Gram-negative bacterial cells.

Two models of therapy were examined. The first was a treatment methodology whereby an acute 2-h infection was allowed to establish prior to administration of phage. At all MOIs of phage there was prolonged survival of *G. mellonella* regardless of whether 10 or 100 bacterial cells were used as the inoculum. Although there was increased survival compared with the control, there was a difference in survival depending on the number of cells in the inoculum. Presumably the 10-fold higher inoculum of 100 cells versus 10 cells meant that the infection had become more established within the 2-h time frame therefore reducing the efficacy of the phage to prolong survival.

The second model examined the ability to prevent infection using prophylactic administration of phage 2 h prior to infection. When compared with the treatment model, prophylactic

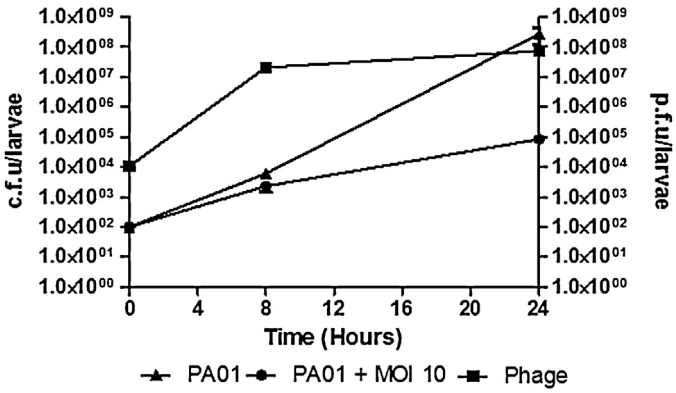

Fig. 3. *In vivo* kinetics of *Pseudomonas aeruginosa* PAO1 infection within *Galleria mellonella* with and without phage treatment. MOI, multiplicity of infection.

administration of phage resulted in greater survival after 24 h at all comparable MOI values. Presumably this increased efficacy was the result of phage being able to distribute throughout the haemolymph over the 2-h period prior to infection, whereas in the treatment model the bacteria will have had opportunity to establish and begin to express toxins. Interesting was the observation of greater survival among *G. mellonella* that received the higher inoculum of 100 cells compared with 10 cells. This may have been due to the higher number of bacterial cells, resulting in an increased chance of bacteria and phage interaction resulting in a more rapid amplification of the phage.

In both models, phage-treated *G. mellonella* eventually succumbed to the infection, resulting in mortality by 30 h post-infection. For this reason, we explored the kinetics of both the *P. aeruginosa* infection as well as the effect of phage on bacterial numbers *in vivo*. The most striking observation was the comparison between numbers of *P. aeruginosa* in the phage-treated and untreated *G. mellonella*. At 24 h the phage had kept the number of *P. aeruginosa* to 1000-fold less than the non-treated *G. mellonella*, but even in the presence of high titres of phage there had still been active growth, and therefore infection, from *P. aeruginosa* over the duration of the experiment. We had previously hypothesised that the reason for eventual mortality was the lack of available phage for clearance. From Fig. 3 it is clear that this is not the case owing to the high titre of phage within the haemolymph, although the MOI had shifted from 100 to <1 by 24 h. This hypothesis was also ruled out by an experiment where *G. mellonella* was given a second dose of phage 4 h after an initial dosing, but there was no difference when compared with the single dose control (data not shown). One possibility for the continual survival of PAO1 in the presence of a high titre of phage was the evolution of phage resistance within the *G. mellonella*. This was ruled out after observation of no bacterial growth when co-cultivating *P. aeruginosa* single colonies, recovered at 24 h after phage treatment, and a suspension of phage cocktail (data not shown). The final explanation for the survival could be the intracellular localisation of *P. aeruginosa*. In these experiments only bacterial numbers within extracted haemolymph were examined. *Pseudomonas aeruginosa* is known to have the ability to invade epithelial cells, which would protect from attack by the phage [18]. This highlights one of the limitations of phage therapy on pathogens that are able to exist and replicate in an intracellular environment. Perhaps combination therapy with antibiotics that can enter host cells, such as a fluoroquinolone or tetracycline, would have aided in clearance, but this was beyond the scope of this study. This potential intracellular survival strategy would also explain why the prevention model showed improved survival compared with the treatment model, whereby the *P. aeruginosa* will have establish within cells before the *G. mellonella* received a dose of the phage.

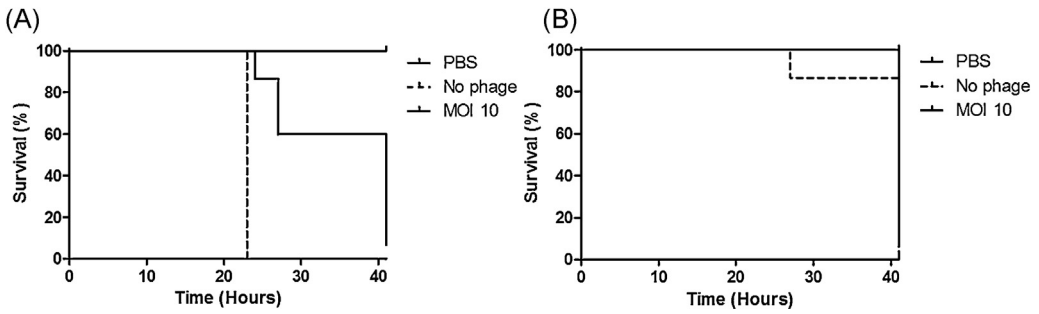

Fig. 4. Kaplan–Meier survival curves of *Galleria mellonella* infected with 10 cells of (A) *Pseudomonas aeruginosa* PA45291 (bacteraemia isolate) or (B) *P. aeruginosa* BC09007 (cystic fibrosis isolate) and pre-treated with phage at a multiplicity of infection (MOI) of 10 at 2 h pre-infection. PBS, phosphate-buffered saline.

Although we hypothesise that the lack of *P. aeruginosa* clearance was due to intracellular localisation, there must have been a degree of extracellular replication of cells within the haemolymph to allow for the observed propagation of the phage over time.

Finally, we looked to demonstrate the effectiveness of the phage model on clinical isolates of *P. aeruginosa*. To do this, the prevention model was repeated with clinical isolates from a bacteraemia and a CF infection. Here the acute isolate resulted in rapid mortality of the *G. mellonella* within 24 h, with 85% survival when given phage at a MOI of 10. Interestingly, the CF isolate was less virulent at 24 h compared with the bacteraemia and PAO1 strains, but 100% mortality was then seen by 40 h. In conclusion, we present data for the use of *G. mellonella* as a simple, robust and cost-effective model for initial examination of *P. aeruginosa*-targeted phage therapy.

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Competing interests

None declared.

Ethical approval

Not required.

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